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Effect of different levels of milkweed (*Calotropis persica*) seed powder on the growth parameters, immunity and gut microbiota of *Oncorhynchus mykiss*

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ABSTRACT

An 8-week feeding experiment was conducted to investigate the inclusion potential of five levels of *Calotropis persica* seed powder (CSP) (0, 10, 20, 30, 40 and 50 g/kg of the basal diet) in rainbow trout (*Oncorhynchus mykiss*) diet, in a completely randomized design. To perform the test, 3600 fries (11.5 ± 3.64 g) were treated for 56 days. According to the results, the increase in milkweed seed powder up to 40 g/kg, resulted in a significant increase in specific growth rate and intestinal lactic acid bacteria count compared to the control ($p < 0.05$). The best results of survival rate, feed conversion ratio, hepatosomatic and gastrosomatic indices were achieved in the treatments receiving 20, 30, 40, and 50 g/kg CSP ($p < 0.05$). Antibacterial activity of skin mucus, lysozyme and alkaline phosphatase showed the highest level in the 40 g/kg treatment ($p < 0.05$). Based on the results, the inclusion of 40 g/kg milkweed seed powder caused positive health effects and could be a suitable herbal feed additive in the rainbow trout diet.

Keywords

Calotropis persica, growth, immunity, gut microbiota, Oncorhynchus mykiss

Abbreviations

CSP: *Calotropis persica* seed powder
FCR: feed conversion ratio
HIS: hepatosomatic index
GaSI: gastro-somatic index
SGR: specific growth rate
ALP: alkaline phosphatase
PTCC: Persian Type Culture Collection
RaRBC: rabbit red blood cells
OD: optical density
CFU: colony forming unit
TVC: total viable count
LAB: lactic acid bacteria
ANOVA: analysis of variance

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Introduction

Despite the positive effects of hormones, antibiotics, vitamins and several other chemicals in the aquaculture industry, their residual and other side effects restrict their use in aquaculture operations (1). Thus, various alternatives have been proposed, including the use of probiotics, prebiotics, synbiotics, herbal medicines, etc. For reasons such as the availability, affordability and accessibility of herbal medicines, they have attracted much attention to aquaculture research, due to the advantages of herbal additives on growth performance, immunity, and appetite promotion (2), inhibitory and/or stimulatory effects in reproduction (3) and their antimicrobial properties (4). The review of the current literature indicates that there is a lot of information about the application of famous herbs such as garlic, rosemary, onion, peppermint, etc. (5) in aquaculture, while other plants with medicinal properties such as milkweed (*Calotropis spp.*) are less studied.

Giant milkweed (*Calotropis persica*) is a wild plant belonging to the *Asclepiadaceae* family (6). It grows in dry habitats (150–1000 mm precipitation per year) (7) and is native of West, North and East Africa, Arabian Peninsula, Southern Asia, and Indochina up to Malaysia (8). The various components of *Calotropis spp.* have different medical and industrial properties reported in literature (9–14). The plant is a rich source of many bioactive compounds which are of medicinal and industrial importance, for example Mohanraj et al, 2009 showed that the leaf extracts of *C. procera* have anti HIV-1 and antimicrobial activity. Other researchers used the latex protein of *C. procera* to prevent the septic shock due to lethal infection by *Salmonella enterica* (9). Also, the antifungal properties of the *C. gigantea* extract are used to treat *Fusarium mangiferae* and floral malformation in mango (12). For industrial applications of *C. gigantea*, oil extraction from seed and biodiesel production can be mentioned (11).

Some researchers have shown that the antibacterial, anti-fungal and anti-viral properties of different parts of *Calotropis Spp.* can be usefully exploited in aquaculture. Recently, *C. persica* ethyl acetate leaf extract was used against shrimp and fish pathogens. Results showed that the extract efficaciously suppressed the bacterial pathogens *Pseudomonas aeruginosa*, *Vibrio harvey*i and *Aeromonas hydrophila*; the fungi *Fusarium Sp.*, and the virus causing the white Spot Syndrome (WSS).

Although previous studies have examined the phytochemical power of different parts of the plant such as leaves, roots, shoots, flowers, fruits and latex, there is a lack of information regarding *C. persica* seed application. Therefore, this study aimed at investigating the effects of *C. persica* seed powder on growth performance, skin mucus immunity, antibacterial activity and gut microbiota, of rainbow trout (*Oncorhynchus mykiss*).

Results

Growth performance

The results of growth performance, survival rate and physiological parameters of *O. mykiss* at the end of the 56 days experiment are shown in Table 1. The results revealed that the dietary administration of different levels of CSP significantly improved SGR, FCR, HSI and survival rate compared to the control ($p < 0.05$). The statistical analysis of the results also indicate that, during the trial, the incremental trend in GaSI paralleled the amount of CSP in the diet. On the other hand, SGR and HSI decreased significantly at 50 g/kg CSP level of inclusion in the diet. ($p < 0.05$). Furthermore, the least FCR was observed in fish receiving 20 g/kg CSP ($p < 0.05$).

Table 1
Weight (Mean ± SD), Condition factor, Specific Growth Rate, Survival Rate, Feed Conversion Ratio, Hepato-Somatic Index, Gastero-Somatic Index of *O. mykiss* fed diets containing different levels of *C. Persica* Seed Powder for 56 days (n = 3) ($p < 0.05$).

	Different levels of dietary CSP (%)					
	0	**10**	**20**	**30**	**40**	**50**
Initial weight (g)	11.40 ± 3.65	11.50 ± 3.64	11.60 ± 3.64	11.70 ± 3.66	11.40 ± 3.64	11.80 ± 3.70
Final weight (g)	33.50 ± 4.97 ^{a}	37.30 ± 3.04 ^{b}	37.30 ± 4.05 ^{b}	41.70 ± 2.18 ^{c}	42.80 ± 3.00 ^{cd}	36.70 ± 5.60 ^{b}
Condition Factor (%)	1.18 ± 0.42	1.17 ± 0.73	1.19 ± 0.52	1.22 ± 0.31	1.24 ± 0.40	1.29 ± 0.32
SGR (%/day)	1.92 ± 0.23 ^{a}	2.12 ± 0.22 ^{c}	2.09 ± 0.23 ^{c}	2.27 ± 0.23 ^{d}	2.36 ± 0.30 ^{e}	2.03 ± 0.20 ^{b}
Survival Rate (%)	82.00 ± 8.66 ^{a}	90.70 ± 8.14 ^{c}	91.70 ± 7.91 ^{e}	90.10 ± 8.06 ^{d}	89.40 ± 8.57 ^{c}	86.80 ± 9.12 ^{b}
FCR	1.71 ± 0.21 ^{c}	1.49 ± 0.10 ^{b}	1.31 ± 0.10 ^{a}	1.38 ± 0.11 ^{ab}	1.47 ± 0.10 ^{b}	1.92 ± 0.11 ^{d}
HIS (%)	1.43 ± 0.20 ^{b}	1.48 ± 0.21 ^{d}	1.45 ± 0.21 ^{c}	1.50 ± 0.20 ^{e}	1.48 ± 0.22 ^{d}	1.37 ± 0.20 ^{a}
GSI (%)	10.13 ± 1.32 ^{a}	11.35 ± 1.37 ^{b}	12.60 ± 1.55 ^{c}	13.41 ± 1.48 ^{d}	14.38 ± 1.90 ^{e}	15.24 ± 1.83 ^{f}

Different superscripts within a row indicate significant differences at $p < 0.05$.
SGR: specific growth rate; FCR; Feed conversion ratio; HIS; hepato-somatic index; GaSI; gastero-somatic index *C. Persica* Seed Powder (CSP)

Skin mucus immunological parameters

Evaluation of the skin mucus parameters (lysozyme activity, ALP activity and dissolved protein content) showed that dietary administration of CSP at any level of inclusion, significantly improved lysozyme activity and ALP activity and dissolved protein compared to the control diet group (Table 2) ($p < 0.05$).

Skin mucus antibacterial activity

The skin mucus of rainbow trout (*O. mykiss*) showed significant antibacterial activity ($p < 0.05$) against *Y. ruckeri*, *A. hydrophila*, *S. iniae*, *S. faecium* and *M. luteus* in all treatments as shown in Figure 1. The highest growth inhibition zone for all tested bacteria was observed in the skin mucus of fish receiving 40 g/kg CSP for 56 days ($p < 0.05$). The results also showed that the highest and lowest antibacterial activity of skin mucus was observed in *A. hydrophila* and *S. faecium*, respectively ($p < 0.05$).

Intestinal bacteria analysis

Table 3 shows the results of Total Viable Count (TVC) and lactic Acid Bacteria (LAB) levels in the intestine of rainbow trout (*O. mykiss*) after 56 days of feeding on different levels of CSP. The count of the

Table 2
Skin mucus dissolved protein (Mean ± SD), alkaline phosphatase activity and lysozyme activity of *O. mykiss* fed with different levels of CSP in the diet for 56 days (n=3) ($p < 0.05$).

	Different levels of dietary CSP (%)					
	0	**10**	**20**	**30**	**40**	**50**
DP (µg/ml)	0.48 ± 0.01 ^{a}	0.50 ± 0.03 ^{a}	0.96 ± 0.02 ^{b}	0.63 ± 0.01 ^{c}	0.67 ± 0.02 ^{d}	0.71 ± 0.02 ^{e}
ALP (µg/ml)	0.58 ± 0.03 ^{a}	0.96 ± 0.02 ^{b}	0.96 ± 0.02 ^{b}	1.51 ± 0.03 ^{de}	1.55 ± 0.03 ^{e}	1.48 ± 0.01 ^{d}
Lysozyme (µg/ml)	1.19 ± 0.08 ^{a}	1.43 ± 0.07 ^{b}	0.96 ± 0.02 ^{b}	2.16 ± 0.03 ^{d}	2.74 ± 0.05 ^{f}	2.28±0.03 ^{e}

Different superscripts (a-f) in each column show significant differences at $p < 0.05$.
DP: dissolved protein; ALP: alkaline phosphatase

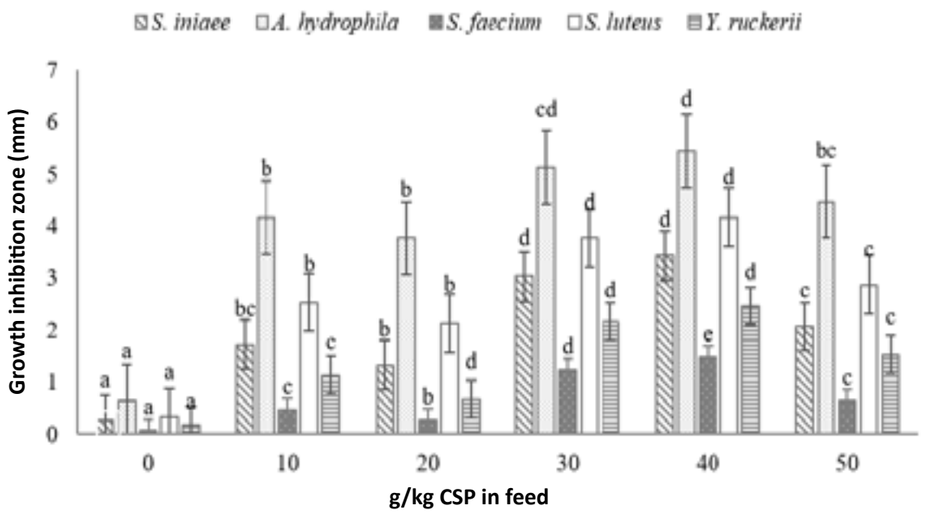

Figure 1
The mean (± SD) of bactericidal activity of juvenile *O. mykiss* fed with different levels of CSP in the diet for 56 days using diffusion disk plates on agar. Bars with different letters are significantly different ($p < 0.05$) against each strain bacteria at three replicates.

intestinal microbiota was reported as colony forming units per gram of intestinal sample (CFU/g). The results indicate that a significant increase in TVC and LAB was observed in the intestinal microbiota of CSP-treated fish, compared to the control ($p < 0.05$). The highest TVC and LAB were shown in the diet containing 40 g/kg ($p < 0.05$). The ratio of LAB count to the total viable aerobic bacteria count reached the highest value in the diet containing 40 g/kg and decreased significantly in the diet containing 50 g/kg ($p < 0.05$).

Table 3
TVC (Mean ±SD; ×10^6), LAB (×10^4) and the ratio of the LAB to TVC (×10^3) in the intestine of *O. mykiss* fed with diets containing different levels of *C. persica* seed powder (CSP) for 56 days (n = 3) ($p < 0.05$).

	Different levels of dietary CSP (%)					
	0	**10**	**20**	**30**	**40**	**50**
TVC (CFU)	4.85 ± 0.49	4.83 ± 0.49	4.75 ± 0.48	4.75 ± 0.48	4.66 ± 0.49	4.79 ± 0.51
LAB (CFU)	3.20 ± 0.35 ^{a}	4.70 ± 0.35 ^{c}	5.00 ± 0.29 ^{c}	5.30 ± 0.31^{cd}	6.10 ± 0.52 ^{e}	3.40 ± 0.39 ^{b}
LAB/TVC	6.60 ± 0.58 ^{a}	9.70 ± 1.00 ^{b}	10.50 ± 1.00 ^{bc}	11.40 ± 0.99 ^{d}	12.70 ± 0.99 ^{d}	6.9 ± 0.52 ^{a}

Different superscripts within a row indicate significant differences at $p < 0.05$.
TVC: Total viable count of aerobic heterotrophic bacteria; LAB: Lactic acid bacteria

Discussion

In recent decades, special attention has been paid to the role of intestinal bacteria regarding the regulation of growth and reproduction in aquatic animals (15). The bacterial communities in the gastrointestinal tract increase the digestibility of proteins, lipids and carbohydrates of the diet by partaking in digestive enzymes (amylase, protease and lipase) secretion (16). LAB are among the main beneficial gut bacteria, and include more than 50 species. These bacteria use carbohydrates as energy and produce lactic acid (16, 17). Some indigestible feed ingredients such as fibers could stimulate lactic acid bacteria propagation and play symbiotic roles (18).

In this study, the inclusion of CSP in the diet of rainbow trout, brought about a significant improvement in growth performance, survival rate and nutritional indices. The findings were similar to that reported by Jayapracas et al. (1997), who investigated the effects of different concentrations of Livol powder in Rohu (Labeo rohita) feed for 112 days. The results indicated that after receiving 2 mg/kg Livol powder, the fish showed the highest SGR and FCR. In the current study, the LAB count increased as a result of the CSP inclusion in the diet, which may suggest that the high fiber content in CSP may contribute to LAB increase. LAB are believed to positively affect growth, FCR and survival rate (19, 20). Therefore, the enhancement of these parameters, may be related to the LAB count increase. However, further studies are needed.

Fish skin mucus is well known to be the first barrier of defense (21), since it contains different immune proteins such as complements, lysozyme, immunoglobulins, protease and lectins (22). Previous studies have demonstrated that fish skin immunity can be improved through the administration of different additives to their diet such as vitamins (23), probiotics (24), prebiotics (25), synbiotics (26) and herbal components (2,27,28). Based on the available information, no studies were found on the effects of CSP on either fish or shellfish skin mucus immunity. The results of the current study indicated that the antibacterial activity in rainbow trout mucus against *S. iniaee*, *A. hydrophila*, *S. faecium*, *S. luteus*, and *Y. ruckerii* increased along with the dietary CSP level of inclusion. However, the mucosal antibacterial activity against all five strains of bacteria decreased with the increment of CSP levels in the diet from 40 to 50 g/kg, an outcome that may indicate that the overdose of CSP supplement can have detrimental effects. Similar results were obtained from other researches (29) which surveyed the effects of Peppermint (*Mentha piperita*) on the skin antibacterial activity of rainbow trout against *Y. ruckeri*. The results presented here also showed that the activity of the immune proteins and total protein content in the skin mucus increased significantly along with CSP presence in the diet. Similar results have been reported in relation to the use of other herbal substances such as garlic (5,30), onion (2,31), peppermint (32), etc., which confirm the increase in the level of blood and mucosal immunity in fish and crustaceans. Given that mucosal lysozyme plays an active role in bactericidal activity, it is likely that mucosal antibacterial activity would be enhanced by stimulated mucus activity (33). This is because the immune proteins in mucus, such as lysozyme and protease, play a vital role in destroying the walls of invasive bacteria (34). The physiological and nutritional status of an aquatic organism plays a significant role in skin mucus immunity such as bactericidal activity (33). In recent studies, it has been found that different parts of milkweed leaf powder (35) latex (36), and seed oil (37) have antibacterial activity. Consequently, it can be assumed that including CSP in the diet causes the increase of the activity of skin mucus compounds (lysozymes, proteases, immunoglobulins, etc). However, the role of gut lactic acid bacteria (LAB) increase on improving mucosal immunity cannot be ignored.

In conclusion, this study we demonstrated the suitability of *C. persica* seed powder as feed additive in rainbow trout *O. mykiss* diet. The best level of inclusion was found to be 40 g/kg CSP added to the diet, capable of improving the mucosal immune responses, the gut microbiota composition and the growth performances.

Material and methods

Fish preparation

A total of 360 healthy rainbow trout [*O. mykiss* (Walbaum 1792)] (average weight 11.50 ± 3.64 g), were obtained from a local fish supplier in Mashhad, (Khorasan Razavi, Iran) and maintained at a density of 200 individuals per cement tank (1 m^3). The fish were acclimatized to the laboratory conditions and kept under observation for clinical health for 14 days prior to starting the experiment. The physico-chemical parameters of water, such as temperature (16.15 ± 3.5˚C), dissolved oxygen (7.90 ± 0.14 ppm) and pH (7.1 ± 0.5), were maintained in accordance with the standard values for rainbow trout culture. All experiments were done according to FUM animal ethics.

CSP preparation

C. persica produces lots of seeds which are flat and brown, and have tufts at one end. Seeds used in this study was obtained from nature (Kerman, Jiroft, Iran). After removing tufts, seeds were dried in an oven at 50°C for 24 h and then ground into powder before adding them to the diets (38).

Diet preparation

A control diet (45.03 g/kg BW, crude protein; 18.40 g/kg, crude lipid; 18.80 J kg, crude energy) was developed by WUFFDA (Windows User-Friendly Feed Formulation; University of Georgia, Georgia, USA) software (39) (Table 4). To prepare the experimental diets, *C. persica* seed powder (CSP) at the inclusion levels of 0, 10, 20, 30, 40 and 50 g/kg for basal diet was employed. CSP was replaced with Carboxymethyl cellulose (CMC). Diets were isonitrogenous, isoenergetic. Feed ingredients were converted into a uniform paste by adding water. Thereafter, the dough was passed through a meat grinder with 2 mm diameter holes (40). The obtained "spaghett" were cut into pellets and dried at 30°C for 24 h (41), and stored at 4°C until use. The experiment was carried out in the form of a completely randomized design. Each diet was fed to three replicate tanks for 56 days and rainbow trouts were fed 2% of their body weight thrice daily.

Evaluation of growth performance and survival rate

Table 4
Composition (g/kg dry matter) of the control diet fed *O. mykiss*

Ingredient	**g/kg (dry-weight basis)**
Fish meal	45
CSP	0
Soybean meal	8
Wheat flour	4.5
Corn gluten	5.6
Corn starch	10.3
Fish oil	9
Canola oil	9
Choline chloride	0.1
Vitamin C (stay)	0.5
Vitamin premix1	1.5
Mineral premix2	1
Dicalcium phosphate	0.5%
Carboxymethyl cellulose	5
Chemical composition of control diet	
Dry matter	870.30
Crude protein (%)	45.03
Crude lipid (%)	18.40
Crude energy (jkg-1)	18.80
Chemical composition of CSP	
Dry matter (%)	95.71
Crude protein (%)	4.75
Crude lipid (%)	20.11
Crude fiber (%)	38.57
Ash (%)	5.04
Nitrogen-free extract	27.29
P (%)	1.54
Ca (%)	2.35
Crude energy (jkg-1)	1.98

Mineral premix contains (mg/kg) Mg, 100; Zn, 60; Fe, 40; Cu, 5; Co, 0.1; I, 0.1; Antioxidant (BHT), 100. 2.Vitamin premix contains (mg kg-1) E, 30; K, 3; Thiamine, 2; Riboflavin, 7; Pyridoxine, 3; Pantothenic acid, 18; Niacin, 40; Folacin, 1.5; Choline, 600; Biotin, 0.7 and Cyanocobalamin, 0.02 (provided from kimia rosh© Gorgan, Iran).

The experimental fish were weighted at the end of the trial on a digital balance with the accuracy of 0.01 g. Survival rate, growth parameters, feed conversion ratio (FCR), hepatosomatic index (HSI) and Gastro-Somatic Index (GaSI) were calculated as follows (41);
Specific Growth Rate (SGR) = [(Ln final weight (g)–Ln initial weight (g))/experiment days] ×100

Feed Conversion Ratio (FCR) = (Feed consumed (g)/Weight gain (g))
Condition factor = (Final weight (g)/Total lenght^3 (cm)) ×100
Survival rate (%) = (Final individual numbers/Initial individual numbers) ×100
Hepato-Somatic Index (HSI) = (Liver weight (g)/Total body weight (g)) ×100
Gastro-Somatic Index (GaSI) = (Gut weight (g)/ Total body weight (g)) ×100

Chemical analyses

Crude protein (Kjeldahl method), crude fat (Soxhlet method), gross energy (parr; electric bomb), crude fiber, ash (electric furnace at 550°C for 6 h), phosphorus (titration with vanadium molybdate) and nitrogen-free extract contents of CSP and control diet (Table 1) were measured according to the standard methods (42).

Skin mucus immunological parameters

At the end of the experiment, the skin mucus samples were collected according to the protocol described by Ross et al. (2000) with some modifications, to monitor antibacterial activity. Briefly, anesthetized fish (clove powder (5 mg/l)) were placed for 2 min in individual plastic bags containing 5 ml of 50 mM NaCl (30 fish per treatment, one by one without pooling). Thereafter, the mucus samples were centrifuged at 1500×g for 10 min at 4°C and the supernatant was stored at -80°C until use.

Protein concentration was determined according to the method of Lowry et al. (1951), using bovine serum albumin as a standard. The absorbance was read using a spectrophotometer (Biochrom, Libra S12) at 750 nm. Skin mucus alkaline phosphatase (ALP) activity was estimated using Pars Azmoun (Pars Azmoon Co, Iran) commercial kit according to the manufacturer's protocol. Lysozyme activity was determined based on the lysis of the lysozyme-sensitive Gram-positive bacterium *Micrococcus lysodeikticus*, according to the protocol described by Demers (43). Alternative complement activity was assayed by using rabbit red blood cells (RaRBC) (44) and based on the guidance released by Neissi et al. (45).

Skin mucus antibacterial activity

The skin mucus antibacterial activity of *O. mykiss* fed CSP was checked out against two gram-negative fish pathogens, *Yersinia ruckeri* PTCC 1888 and *Aeromonas hydrophila* ATCC 7966 and three gram-positive bacteria *Streptococcus iniae* PTCC 1887, *Streptococcus faecium* ATCC 19434 and *Micrococcus luteus* PTCC 1169, using the disk diffusion method (46). 0.1 ml of each bacteria (1 ×10^5 CFU ml/1;OD600) was mixed with the nutrient agar medium (Merck, Germany). Paper discs (6 mm diameter) were inoculated with 150 ml of the mucus sample and kept for 20 min to allow the mucus placed on the medium to be absorbed. Plates were incubated for 24 h at 37°C. Finally, the diameter of the inhibition zone was measured by Image J (1.45s) software.

Intestinal bacterial analysis

At the end of the experiment, the fish were anesthetized with clove powder (5 mg/l), disinfected with 70% ethanol and the whole gut was removed (47). Enumeration of the total viable count (TVC) of heterotrophic bacteria and *lactobacillus* bacteria (LAB) in the intestine was assessed using 1 g of posterior intestine sample. The samples were homogenized in 9 ml normal sterile saline solution (0.90% w/v of NaCl) and dilutions prepared to 10^8. Then, 0.1 ml of the saline solution was spread over duplicate plates of plate count agar (for total heterotrophic bacteria) and MRS agar (for LAB). The plates were incubated at room temperature for 72 h. The bacterial colonies were counted in each sample based on the colony forming unit (CFU/g) (colony count × dilution-1= CFU / g intestine) (48).

Statistical analysis

All percentage data were transformed using the arcsine method. Levene's test was used to confirm the homogeneity of variance, while the Kolmogorov-Smirnov test was used to determine the normality of data (Zar, 1999). The data were analyzed using one-way ANOVA and Duncan's multiple range test was applied to determine any significant differences among the treatments ($p < 0.05$).

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Author Contributions

Conceived and designed the experiments: O.S., Performed the experiments: O.S., Analyzed the data: H.A.M., Research space and equipment: O.S., H.A.M., Contributed reagents/materials/analysis tools: O.S., M.P., Wrote the paper: H.A.M.

Conflict of Interest

The authors declare that they have no competing interests.

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